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54 USE OF ISOFLAVONES AND/OR EXTRACTS FROM THE AFRICAN PLUM TREE IN PHARMACY, COSMETICS AND AS A FOOD ADDITIVE.

57 The present invention relates to the use of at least one product chosen from the group constituted by the isoflavones, the extracts of the African plum tree, and their mixtures, for the preparation of a composition intended to inhibit the activity of 5α-reductase. This use makes it possible to obtain a noteworthy inhibitory effect on the activity of 5α-reductase, thus providing a new answer for the treatment of dermatological pathologies and/or disorders linked to a congenital or acquired hyperactivity of 5α-reductase, and in particular for the treatment of prostatic hypertrophy, prostatic adenoma, acne, hyperseborrhea, alopecia and hirsutism. The invention also relates to methods of cosmetic treatment, in particular of oily skin, as well as the use of the said described products as additives in food for human beings and/or animals.

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The present invention relates to the use of at least one product chosen from the group constituted by the isoflavones, the extracts of the African plum tree, and their mixtures, for the preparation of a composition intended to inhibit the activity of 5α-reductase, in particular for the treatment of prostatic hypertrophy, prostatic adenoma, acne, hyperseborrhea, alopecia and hirsutism.

The invention also relates to methods of cosmetic treatment, particularly of oily skin, as well as to the use of the described products as additives in food for human beings and/or animals.

5α-Reductase is an NADPH-dependant microsomial enzyme which exists in the form of two isoenzymes synthesized starting from two different genes.

The type 1 isoenzyme of 5α-reductase is found mainly in the liver and the skin, more particularly in the sebaceous glands of the non-genital skin and of the scalp, and appears at puberty. The type 2 isoenzyme is predominant in the prostate and in the skin of the differentiated genital areas: the genital region and the beard, and plays a part in sexual differentiation. The distribution of the type 1 and 2 isoenzymes of 5α-reductase in the skin and cutaneous appendages in man can be illustrated by Table 1 below.

There are a number of pathologies for which a congenital or acquired hyperactivity of 5α-reductase is completely or mainly responsible for the problems observed.

For example, in man, this 5α-reductase enzyme, mainly localized in the genital tissues and in the skin, catalyzes the hydroxylation of testosterone to 5α-reductase dihydrotestosterone (DHT). Since DHT is a much more active androgen than testosterone (about two times more), the effects of the latter are amplified in the tissues where DHT is produced. Too high an activity of the 5α-reductase thus causes contents of androgen in the form of DHT in the prostate that are too high, resulting in an over-stimulation of the latter expressed as an undesirable growth that can lead to a prostatic hypertrophy pathology, or even prostatic adenoma, most often requiring surgical intervention.

Table 1: Distribution of type 1 and 2 isoenzymes of 5α-reductase in the skin and cutaneous appendages in man.

		H5-α r1	H5-α r2
EPIDERMIS	Basal layer	++	+
	Spinous layer	+	++
	Granular layer	+	-
	Corneal layer	-	-
DERMIS	Fibroblasts	++	-
SEBACEOUS GLANDS	Basal cells	++	+
	Glandular cells	++	-
ECCRINE SWEAT GLANDS	Excretory channel	-	-
	Secretory cells	++	-
	Myoepithelial cells	++	+
HAIR FOLLICLE	Dermal papilla	+	+?
	Cells of matrix	++	+
	Internal epithelial sheath	±	+++
	External epithelial sheath	++	-
	Arrector muscle	+	-

Other pathologies of the dermatological type can be observed in men or women as a result of a hyperactivity of 5α-reductase, that is, in particular acne, hirsutism or alopecia.

In the skin, the activity of 5α-reductase is greater in the sebaceous gland than in the other structures. Moreover, the sebaceous glands display a greater 5α-reductase activity than that of other cutaneous regions. Consequently, the physiological sebaceous secretion level seems to be closely linked to the activity of this enzyme.

In the acne patient, there is a hyperactivity of 5α-reductase. More than just an increase in the serum levels of androgens, it is an increase in DHT precursors, a principal factor in the sebaceous function that participates in acne.

Oily skin, or seborrhea, besides its unattractive appearance, constitutes a terrain on which complications can arise. It affects regions where the sebaceous glands are numerous, and mainly results in an androgenic overstimulation of the sebaceous production by these specific glands. Hyperseborrhea participates in the appearance of the lesions of acne vulgaris.

In the scalp, the type 1 5α-reductase isoenzyme is found in the sebaceous glands as well as in the hair follicle. The type 2 5α-reductase isoenzyme is primarily localized in the internal epithelial sheath, as well as in the dermal papilla of the hair. However, this last localization remains to be determined exactlv.

Androgenic alopecia, the physiopathogeny of which is very similar to that of acne, is the most frequent of the alopecias and undoubtedly the one where the demand for treatment is the strongest. 5α-Reductase seems to play an essential role in this pathology. In fact, men suffering from a genetic deficit in the type 2 5α-reductase isoenzyme do not develop androgenic alopecia.

Taking the above into consideration, the research is oriented towards the perfecting of 5α-reductase inhibitors. Certain steroids, such as progesterone, have been tested in this regard, but its rapid metabolization renders it inefficacious *in vivo*. To be active, the 5α-reductase inhibitor must be sufficiently stable to block the activity of the enzyme *in vitro*. Finasteride, a competitive steroidal inhibitor, fulfills this condition, but it is more active on the type 2 isoenzyme than on the type 1 isoenzyme, and these two enzymes have only 50% homology over the sequence of their amino acids. It is therefore above all in benign prostatic hyperplasia that finasteride has already been tested.

Moreover, the extract of *Serenoa repens* is also known as a reference as a 5α-reductase inhibitor, the extract of *Serenoa repens* having the advantage, relative to finasteride, of a natural origin as a plant extract, permitting a better comparison for the products tested, likewise of natural origin. *Serenoa repens*, also known by the designation *Sabal serrulatum*, is a small palm tree that can be found in the United States (Florida), in North Africa and in Spain.

Surprisingly and unexpectedly it has now been found that the use of certain compounds of plant origin make it possible to obtain a remarkable inhibitory effect on the activity of 5α-reductase, thus providing in particular a new answer for the treatment of the dermatological pathologies and/or disorders mentioned above.

The present invention thus relates to the use of at least one product chosen from the group constituted by the isoflavones, the extracts of African plum tree, and their mixtures, for the preparation of a composition intended to inhibit the activity of 5α-reductase.

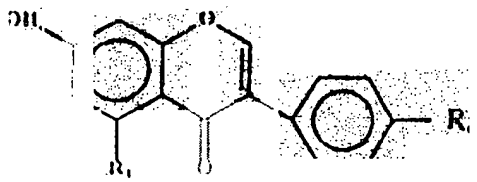
According to the invention, the expression "and their mixtures" above of course encompasses in particular mixtures of isoflavones, mixtures of extracts of the African plum tree, or also mixtures of isoflavone(s) and extract(s) of the African plum tree.

In particular, the use in accordance with the invention is characterized by the fact that the composition is intended to inhibit the type 1 isoenzyme and/or the type 2 isoenzyme of 5α-reductase.

The isoflavones that can be used in accordance with the invention can be obtained by chemical synthesis or are natural substances extracted from natural products, particularly from plants.

There is a distinction between aglycon forms of the isoflavones and the glycosylated forms of the latter. These different forms are illustrated by the following formulas.

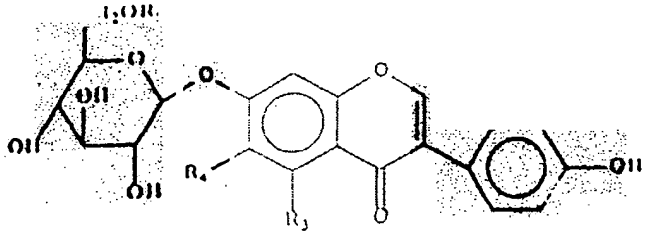
Aglycon forms, of formula:



in which R_1 and R_2 [sic] represent:

R_1	R_2	R_6	Name of compound
H	H	OH	Daidzein
OH	H	OH	Genistein
H	OCH_3	OH	Glycitein
H	H	OCH_3	Formononectin
OH	H	OCH_3	Biochanin A

Glycosylated forms, of formula:

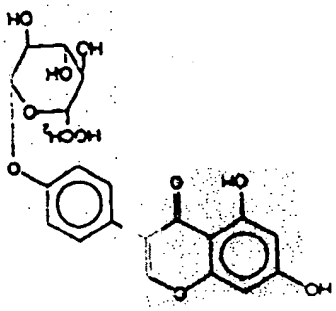


in which R_3, R_4 and R_5 represent:

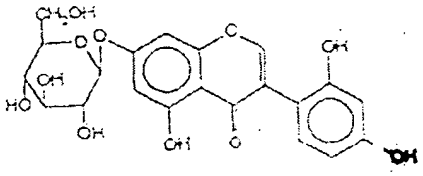
R_3	R_4	R_5	Name of compound
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	Acetyldaidzin
OH	H	C OCH_3	Acetylgenistin
H	OCH_3	C OCH_3	Acetylglycitin
H	H	$COCH_2COOH$	Malonyldaidzin
OH	H	$COCH_2COOH$	Maonylgenistin
H	OCH_3	$COCH_2COCH$	Malonylglycitin

Also to be cited are:

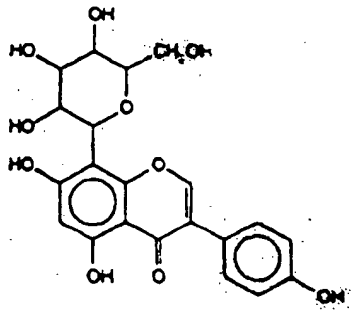
- genistein-4'-O-glucoside of formula:



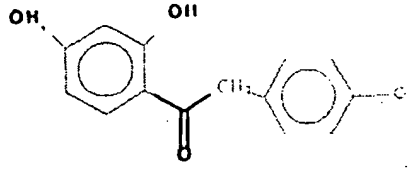
- 2'-hydroxygenistein-7'-O-glucoside of formula:



- genistein-C-8-glucoside of formula:



- 2,4,4'-trihydroxydeoxybenzoin (THB) of formula:



The glycosylated forms of the isoflavones are hydrolysed under the action of beta-glucosidases. The glycosyl (daidzin and genistin) and acyl forms are the most abundant. Acid chemical or enzymatic hydrolysis can transform these conjugated forms into the more absorbable daidzein and genistein.

The use in accordance with the invention is characterized by the fact that the product is chosen from among the synthetic or naturally occurring isoflavones of the group constituted by genistin, daidzin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin, malonylglycitin, 2,4,4'-trihydroxydeoxybenzoin (THB), daidzein, genistein, glycitein, formononectin, biochanin A, genistein-4'-O-glucoside, 2'-hydroxygenistein-7-O-glucoside, genistein-C-8-glucoside, and their mixtures.

According to the invention, it is more particularly preferred to use a product chosen from the group constituted by genistin, genistein, and their mixtures.

No natural sources are known that are as rich in isoflavones as soybeans. The methods of purification of the isoflavones from soy products, in particular the seeds, soybean sprouts (or germinated soybeans), soybean milks including grinds and molasses, and fermented products (in particular tofu and tempeh) are well known to those skilled in the art.

Table 2 below illustrates the isoflavone content (micrograms/gram) in soy beans harvested in Iowa (Wang and Murphy, "Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year and location," J. Agric. Food Chem. 42, 1674-1677 (1994)).

Table 2: Example of isoflavone content in soybeans

Aglycon forms	µg/g dry extract
Daidzein	7 - 60
Genistein	17 - 56
Glycitein	20 - 24
Glycosylated forms	
Daidzin	180 - 780
Genistin	325 - 850
Glycitin	53 - 70
6"-O-Malonyldaidzin	121 - 410
6"-O-Malonylgenistin	290 - 958
6"-O-Malonylglycitin	61 - 72
6"-O-Acetyldaidzin	tr
6"-O-Acetylgenistin	2 - 10
6"-O-Acetylglycitin	23 - 36

The germinated soybean is the soybean source that is richest in isoflavones.

With regard to soy milk, this is traditionally obtained in the warm by grinding the beans after dehusking, in alkaline medium. A heat treatment is effectuated to inhibit the anti-trypsin factors.

The quantities of isoflavones present in soybean milks are variable. Table 3 below is an illustration of the isoflavone content of soybean milks.

Table 3: Example of isoflavone content in soybean milks

Aglycon forms	µg/g dry extract	mg/l soybean
Daidzein	18	1.4
Genistein	19	1.5
Glycitein	10	0.8
Glycosylated forms		
Daidzin	410	33
Genistin	710	57
Glycitin	65	5
6"-O-Malonyldaidzin	690	55
6"-O-Malonylgenistin	871	70
6"-O-Malonylglycitin	39	3
6"-O-Acetyldaidzin	22	18
6"-O-Acetylgenistin	820	66
6"-O-Acetylglycitin	89	7

The ground material or molasses resulting from the production of soy milk also represent a source of soybean isoflavones.

By "extracts of soybean isoflavones" are thus understood in accordance with the invention the extracts of soybean isoflavones obtained from the different soybean products described above (beans, soy bean sprouts or germinated soybeans), soybean milks including ground material and molasses and fermented products (in particular tofu and tempeh), extracts which have possibly been purified and/or concentrated to increase their isoflavone content by methods well-known to one skilled in the art

The use in accordance with the invention is thus also characterized by the fact that the said product is chosen from among the isoflavone extracts from soybeans.

Cited in particular may be the extract of soybean isoflavones marketed by the Nutrinov company under the name Genosten 4000®. This is a soluble soybean extract enriched in isoflavones obtained after concentration by successive evaporations of soybean molasses. This process does not have recourse to any organic solvent. The analytical data on this product will be found in the examples below.

The lupin (*Lupinus*) is likewise an important natural source of isoflavones. In particular, the "yellow lupin" variety may be cited, in the stems of which have been identified in particular the glycosylated isoflavones genistin, 2'-hydroxygenistein-7-O-glucoside, genistein-4'-O-glucoside and genistein-C-8-glucoside, the respective formulas of which are described above (Rafal Franski et al., "Application of mass spectrometry to structural identification of flavonoid monoglycosides isolated from shoot of lupin (*Lupinus luteus L.*)", Acta Biochimica Polonica, vol. 46, No. 2/1999, 459-473).

The use in accordance with the invention is also characterized by the fact that the product is chosen from among the extracts of lupin isoflavones, that is, by analogy with the above definition for the extracts of soybean isoflavones, among the extracts of isoflavones that can be obtained from lupin products.

Extracts of the African plum tree, or "*Pygeum africanum*," are well known to one skilled in the art. They come mainly from the bark of the African plum tree. They are sterolic extracts of the African plum tree, such as those marketed by the Euromed and Indena companies under the respective designations "*Pygeum*" and "*Prunus Africana*." Some physico-chemical characteristics of these two extracts are given in the examples below.

Used in particular in accordance with the invention is the product as described above, in the form of isoflavone(s) from soybeans, the extracts of the African plum tree, or their mixture, in a proportion of between about 0.001 and about 100% by weight (use in pure form of the said product possible), preferably between about 0.01 and about 70% by weight, and still more particularly between about 0.1 and 10% by weight, relative to the total weight of the composition.

The composition prepared by the use in accordance with the invention can in addition include a pharmaceutically, dermatologically or cosmetically acceptable excipient. Any excipient suitable for the galenical forms known to one skilled in the art can be used, with a view to administration by the topical, oral, enteral or parenteral route, in particular the rectal route.

In particular, this excipient can be adapted to obtain a composition in the form of an oily solution, a water-in-oil emulsion, an oil-in-water emulsion, a microemulsion, an oily gel, an anhydrous gel, a cream, a vesicular dispersion, microcapsules, or microparticles, or also gelatin capsules or soft gelatin or vegetable capsules.

Preferably, an excipient is used that is suitable for administration by the external topical route or by the rectal route.

The advantageous effect of inhibiting the activity of 5α-reductase provided by the use in accordance with the invention makes it possible to design the composition thus prepared for therapeutic, in particular dermatological and cosmetic treatments.

Thus, the use in accordance with the invention is characterized by the fact that the composition is intended for the treatment of cutaneous pathologies and/or disorders linked to a congenital or acquired hyperactivity of 5α-reductase.

In particular, the use in accordance with the invention is characterized by the fact that the composition is intended for the treatment of prostatic hypertrophy.

In addition, the use in accordance with the invention is characterized by the fact that the composition is intended for the treatment of prostatic adenoma.

The use of an excipient suitable for administration by the rectal route as described above can be particularly envisaged for these treatments of prostatic hypertrophy and/or adenoma.

The use in accordance with the invention is likewise characterized by the fact that the composition is intended for the treatment of acne.

The use in accordance with the invention is likewise characterized by the fact that the composition is intended for the treatment of hyperseborrhea.

The use in accordance with the invention is likewise characterized by the fact that the composition is intended for the treatment of alopecia.

The use in accordance with the invention is likewise characterized by the fact that the composition is intended for the treatment of hirsutism.

The present invention also has as its subject a cosmetic method for the treatment of oily skin, characterized by the fact that to the skin is applied a cosmetic composition containing at least one product chosen from the group constituted by the isoflavones, the extracts of the African plum tree, and their mixtures, as described above.

The invention moreover has as its subject a cosmetic method of treatment for hair loss, characterized by the fact that to the scalp is applied a cosmetic composition containing at least one product chosen from the group constituted by the isoflavones, the extracts of the African plum tree, and their mixtures, as described above.

Finally, the invention also has as its subject a cosmetic method of treatment for excess hair, characterized by the fact that to the zones of the skin displaying this excess hair is applied a cosmetic composition containing at least one product chosen from the group constituted by the isoflavones, the extracts of the African plum tree, and their mixtures, as described above.

In fact, unlike hormonal medical treatments, these last two cosmetic treatment methods make it possible to improve the appearance by visibly reducing the unattractive phenomena of hair loss linked with alopecia and the phenomena of excess hair linked with hirsutism.

According to a preferred method of execution of these cosmetic treatment methods, the said product is present in the composition in a proportion of between about 0.001 and about 100% by weight (use in pure form possible, without excipient), preferably between about 0.01 and about 70% by weight, and still more particularly between about 0.1 and 10% by weight, relative to the total weight of the composition.

Advantageously, the cosmetic composition applied by the cosmetic method in accordance with the invention contains in addition at least one cosmetically acceptable excipient as described above.

Finally, the invention also has as its subject the use of at least one compound chosen from the group constituted by the isoflavones, the extracts of the African plum tree, and their mixtures, as described above, as an additive in a food for human beings and/or animals. This use in food is preferably characterized by the fact that the said additive is present in the food in a proportion of between about 0.001 and about 100% by weight, preferably between about 0.01 and about 70% by weight, and still more particularly between about 0.1 and 10% by weight, relative to the total weight of the food.

The following examples are intended to illustrate the present invention and should in no case be construed as limiting its scope.

Unless otherwise indicated, the percentages indicated in the following examples are percentages by weight.

Example 1: Evaluation of the inhibitory effect on the activity of 5α-reductase by measurement of the levels of 5-dihydrotestosterone formed from testosterone by the DU145 cells.

1. Materials and methods

1.1. Materials

The prostatic cells DU145 come from a tumor line obtained from a prostatic carcinoma (No. ATCC HTB 81). The MEM medium (ref. 0410265), glutamine and gentamycin come from Gibco. The fetal calf serum (FCS) comes from DAP and is used in decomplemented form (45 mm at 56°C). The plastic items used for the culture (boxes and plates) come from Costar. The testosterone comes from Sigma.

1.2. Method

1.2.1. Preparation of ranges of products

A 10 mg/ml stock solution in ethanol is prepared from each of the products tested.

The range of concentrations used for the experiments is as follows: 0, 5, 10, 50, 100 and 500 micrograms/ml. (Dilution carried out in the culture medium.) Since the volume of extract added per well is 20 microliters/well, the solutions to be prepared are concentrated 50-fold.

Preparation of the Testosterone

A 10 mM stock solution of testosterone is prepared in ethanol. At the time of use, this solution is diluted to 1:1000 in the culture medium and 10 microliters are added per well.

1.2.2. Experiment on the inhibition of the 5α-reductase of DU145.

The DU145 prostate cells are cultivated at 37°C, 5% CO_2, in an MEM medium containing glutamine (2 mM), gentamycin (50 micrograms/ml) and 10% FCS. Their sub-culture level is 1:10.

Before starting the experiment, the cells are cultured in 6-well plates at a rate of 2.10^5 DU145 per well/1 ml medium containing only 1% FCS. The cells are kept for 3 days at 37°C, 5% CO_2. On the day of the experiment, the culture medium contained in the wells is eliminated and replaced by new medium containing 1% FCS. The testosterone (0.1 micromolar, final) as well as the extracts at the different concentrations are added to the medium at a rate of 10 and 20 microliters/well, respectively. (The "control" wells correspond to cells incubated in the presence of testosterone and one equivalent of ethanol. This makes it possible to subtract the effect of the solvent on the cultures and to determine the percentage of DHT formed in the absence of inhibitor.) The cells are then incubated at 37°C, 5% CO_2. After 3 hours, the supernatant liquids of the culture are collected and frozen at -80°C until assay.

Measurement of level of DHT formed.

Principle: extraction of lipophilic products with ether, concentration of DHT samples by affinity chromatography and radio-immunological assay.

Preparation of samples

- After having swirled the withdrawn samples, introduce the samples into "SEPEX" flasks.
- Add to each tube 0.1 ml radioactive solution "3H-Yld" (for the evaluation of the extraction yield). Stopper the flasks, swirl them one by one.
- Leave to stand for 30 min at ambient temperature. Then swirl each flask again.
- Add to each flask: 5 ml ethyl ether.
- Stopper the flasks and agitate them energetically by hand. Leave to decant for a few minutes.
- Freeze the aqueous phases at -30°C for at least an hour.
- Collect the ether phase in a corresponding 5-ml borosilicate test tube.
- Evaporate the ether phase completely using an evaporator + water bath system at 37°C.

Separation of DHT

• Preparation of columns: Prepare the columns in 5-ml glass culture pipettes with 10 cm chromatolith A

• Rinsing of columns: 3 ml pure iso-octane combitips (3 times), letting it flow by simple gravity.

• Elution of dry ether extracts.

- Each dry extract is taken up in 1 ml pure isooctane, swirl vigorously. Wait 15 min at ambient temperature. Re-swirl.
- When the 3 ml isooctane (washing of columns) are eluted, decant the dry ether extracts taken up in isooctane on to the column. Allow to elute.
- Rinse each "dry extract" tube with 1 ml pure isooctane combitips, swirl vigorously. Wait 15 min at ambient temperature. Reswirl and decant into column as previously.
- Wash with 4 ml pure isooctane.

• Collection of DHT

- Prepare the elution solvent (6% mixture of isooctane/ethyl acetate: 94/6 (v/v))
- Elute with 6 ml (pipette) of this mixture
- Collect the DHT eluate in marked 5-ml borosilicate test tubes.

Treatment of DHT eluate: Evaporate the solvent from the eluate using the evaporator-water bath system (37°C)

RIA assay

- Distribution protocol: Take up the samples in 0.5 ml RC buffer, the blank in 1 ml Rcet buffer, the controls in 0.5 ml RC buffer. Place in the furnace at 37°C for 15 min. Agitate the tubes again on leaving the furnace (1 min).

Into the marked 5-ml glass hemolysis tubes place in order:

* **Buffer**: Total Activity (TA): 0.7 ml RC buffer. Non Specific Activity (N): 0.2 ml RC buffer. Range: only the point 0 of the range (marked B_0) includes 0.1 ml RC buffer,

* at 37°C (1000 at 7.8 pg/tube): 0.1 ml of the respective **standard solution**.

* 0.1 ml **dry extract** taken up in the buffer

– then, distribute the **antiserum**: 0.1 ml into all the tubes except TA and N.

– Then, distribute the **"3HD" assay solution**: 0.1 ml into all the tubes.

– Swirl and cover with a parafilm.

– Incubation at 4°C for a minimum of 1.5 hrs (24 hrs maximum).

- Preparation of charcoal-dextran: Put the suspension of charcoal-dextran into a beaker, then into an ice-water bath at 4°C for at least 1.5 hrs.

- Purification yield of DHT

- Into 6 small scintillation vials (3 per series) deposit: 0.4 ml RC buffer + 0.1 ml of the "3H-Yld" solution (flask from first day in refrigerator). Blanks: Put 0.5 ml reconstituted dry extract for the blank. Samples and controls: put 0.25 ml RC buffer and 0.25 ml extract.

- Add 5 ml scintillation liquid to all vials.

Separation of free DHT from that bound to the antibody

– Put the charcoal-dextran suspension under magnetic stirring in an ice-water bath.

– Add 0.5 ml charcoal-dextran to all tubes except TA in 2 min maximum.

– Swirl, replace tubes in the ice water. Wait exactly 10 min. Centrifuge at 4°C, 3400 rpm, for 11 min.

– Pipette 0.5 ml of each supernatant liquid (including TA) into a small counting vial.

– Add 5 ml scintillating liquid. Agitate, allow to equilibrate for 30 min at ambient temperature.

– Set to count 2 min with the β counter (Beckman, LS 6000 SE).

2. Analytical reports of products tested

2.1. The extract of soybean isoflavones tested, called Genosten 4000, was supplied by the Nutrinov company

Description: Soluble soybean extract enriched in isoflavones, obtained by a physical extraction technique without use of organic solvents.

Physico-chemical characteristics:

Density	400 g/l
Moisture	<5%
pH (4% aqueous solution)	8
Solubility in water	100%

Composition:

Proteins	11%
Lipids	<0.5%
Carbohydrates	
Sodium	1500 mg/100g
Calcium	< 100 mg/100g
Potassium	5500 mg/100g
Phosphorus	300 mg/100g

Isoflavone composition:

Total isoflavone content	4000 +/- 200 mg/100g
Typical profile:	
Daidzin	
Genistin	280
Malonyldaidzin	900
Malonylgenistin	2080
Daidzein	30
Genistein	10

2.2. The extract of *Pygeum africanum* tested was supplied by the Euromed company.

Description: Lipidosteroid extract of *Pygeum africanum* obtained from pygeum bark

Physico-chemical characteristics:

Appearance	Viscous dark brown paste with characteristic odor
Solubility	Insoluble in water, soluble in chloroform
Loss on drying	3% max

Ash	0.4% max
UV absorption	Maxima at 242, 282, and 320 nm
Chemical composition	
Acid number	39 mg KOH/g
Fatty acid composition (%)	
C12:0	0.3
C16:0	44.6
C18.0	5.6
C18:1	36.6
C18:2	9.8
C18:3	0.6
Unsaponifiables content	17.6%
Sterols	14.7

3. Results - Evaluation of the conversion of the testosterone to 5-dihydrotestosterone by the DU-145 cells - Determination of IC50

Table 4

Product tested	IC50 (µg/ml)
Soybean isoflavone extract (Genosten 4000)	71
Extract of *Pygeum africanum*	203
Serenoa repens	60

4. Conclusions

The extract of *Serenoa repens*, chosen as the reference substance, inhibits the activity of the 5-alpha-reductase. This results thus validates the test.

The extract of soy isoflavones, with a small amount of isoflavones (4%) is as active as the extract of *Serenoa repens*, chosen as the reference substance inhibiting the activity of 5-alpha-reductase.

The extract of *Pygeum africanum* tested is active in the inhibition of 5-alpha reductase.

Example 2: Evaluation in vitro of the activity of 5α-reductase on the conversion of testosterone to 5α-dihydrotestosterone in cultures of normal human dermal fibroblasts.

Abbreviations used below:

^3H:	tritium
	thin layer chromatography
Ci:	Curie
DMSO:	dimethylsulfoxide
	designation given to a standard culture medium
FCM:	fibroblast culture medium
MEM:	designation given to culture medium, *Minimum Essential Medium*
FIM:	fibroblast incubation medium
Rf:	relative retention factor
FCS:	fetal calf serum
5α-DHT	5α-dihydrotestosterone

It is proposed to evaluate the effect of products such as an extract of soybean isoflavones (Genosten 4000, described above), genistein and genistin (purified isoflavones, commercial products from Sigma), an extract of *Pygeum africanum*, an extract of *Serenoa repens* chosen as a reference, on the activity of 5α-reductase. An in vitro model of cultures of normal human dermal fibroblasts was used.

1. Materials and methods

1.1. Test products, reference product, and reagents

The products to be tested were provided by EXPANSCIENCE, and were preserved at +4°C until use

The radioactive testosterone (labeled with tritium at position 1, 2, 6, and 7, specific activity 79 Ci/mmol) was provided by AMERSHAM; the unlabeled testosterone was supplied by SIGMA.

The analytical grade reagents came from SIGMA, MERCK, BDH, ALDRICH or CARLO ERBA unless otherwise indicated.

1.2. Testing system

The fibroblast culture medium (FCM) was constituted of MEM/M199 (3:1, v/v) with the addition of penicillin (50 IU/ml), streptomycin (50 µg/ml), sodium bicarbonate (0.2%, w/v) and VCS (10%, v/v).

The testing system was constituted of normal human dermal fibroblasts cultivated in a monolayer. The fibroblasts were isolated from a residue from abdominal plastic surgery carried out on a 51-year-old woman (BIOPREDIC subject No. 10013). The cells were used at the fifth passage; they were cultivated up to the confluence of the monolayers in the FCM medium at 37°C in a humid atmosphere containing 5% CO_2.

1.3. Preparation of products and incubation with the test system

The fibroblast incubation medium (FIM) was constituted of FCM with the addition of tritiated testosterone (1.6×10^{-7} M, or 6.32 µCi/ml) and unlabeled testosterone (3.84×10^{-6} M).

The products to be tested and the finasteride were taken up in DMSO before being diluted in the incubation medium. The final concentration in DMSO was kept constant and equal to 1% (v/v) in each dilution of test products and of reference product.

Time scale:

T_{-3} T_0 T_{22}

• Hours

⇓⇑ ↓↑ ✢

⇓: elimination of FCM medium

⇑: pre-incubation of test products and of reference product prepared in the FCM medium

↓: elimination of FCM mediums containing the test products or the reference product

↑: incubation of the test products and of the reference product prepared in the FIM medium

✢: determination of the activity of 5α-reductase

The fibroblast cultures were pre-incubated in the presence of the test products or the reference product for 2 hours before the addition of the substrate, testosterone. For this step, the test products and the reference product were prepared in the FCM medium.

After the pre-incubation, the fibroblast cultures were incubated in the presence of the test products or the reference product prepared in the FIM medium for 22 hours (or 24 hours, indicated with the results) at 37°C in a humid atmosphere containing 5% CO_2. Control cultures were incubated in the FIM medium in the absence of test products and reference product. "DMSO control" cultures were incubated in the FIM medium containing 1% (v/v) DMSO.

Each experimental condition was tested in triplicate.

1.4. Evaluation of effects

After the incubation period, the cells were subjected to ultrasound action in the FIM medium. The cellular lysates thus obtained were extracted with dichloromethane. After evaporation, the residues were taken up in methanol and were deposited on $60F_{254}$ silica plates (MERCK reference 5554).

Non-radio-tagged standards, testosterone, 5α-testosterone, and androstenedione, were deposited on each of the plates.

The migration solvent was a mixture of dichloromethane and ether (7:3, v/v). At the end of the migration, the silica plates were read using a BERTHOLD radioactivity scanner.

The non-radio-tagged standards were visualized by spraying 5% (v/v) sulfuric acid on to the chromatography plates then heating to 100°C for 10 minutes.

Comparison of the Rf (relative retention factor) determined for the standards with those obtained for the different radioactive metabolites permitted the identification of the latter.

The metabolization of the testosterone to 5α-dihydrotestosterone under the different experimental conditions was calculated: the results (areas of the 5α-dihydrotestosterone peaks counted by the BERTHOLD scanner) were expressed in pmoles of 5α-dihydrotestosterone formed per culture well. They were also expressed as the percentage of the 5α-reductase activity present in the "DMSO control" group.

2.5. Treatment of data

The groups of data (control group and treated groups) were treated by a single factor variance analysis (ANOVA 1, $p<0.05$), followed by a DUNNETT test ($p<0.05$). The effect of the test products and of the reference product was compared with the "DMSO control" group. The effects of the test products were compared among themselves by a two-factor variance analysis (ANOVA 2, $p<0.05$, factor 1 = concentration and factor 2 = treatment).

2. Results and discussion

Refer to paragraph 3 below for the detailed tables of results.

2.1 Genistin and genistein

In the "DMSO control" samples (0.1% v/v), the rate of metabolization of the testosterone was 11.40 +/- 0.75 pmoles of 5α-DHT formed in 24 hours per culture well (Table in paragraph 3.1.). This rate was in conformity with the results already obtained in the laboratory.

Genistin, tested at 0.1, 1 and 10 µg/ml, had no significant ($p<0.05$) inhibitory effect on the activity of 5α-reductase. At 100 µg/ml, this product significantly ($p<0.05$) inhibited the activity of the 5α-reductase by 32%.

Genistein, tested at 0.1, 1 and 10 µg/ml, significantly ($p<0.05$) inhibited the activity of the 5α-reductase, by 32%, 33% and 31% respectively. At 100 µg/ml, it significantly ($p<0.05$) inhibited the activity of 5α-reductase by 61%.

In conclusion, genistein is clearly more active than genistin.

2.2. Extract of soybean isoflavones

In the control cultures, the rate of metabolization of testosterone was 9.71 +/- 0.77 pmoles of 5α-DHT formed in 22 hours per culture well (Table in paragraph 3.2.). This rate was in conformity with the results already obtained in the laboratory.

The extract of soybean isoflavones, tested at 10 and 100 µg/ml, inhibited the activity of the 5α-reductase by 22 and 17%, respectively.

The extract of *Serenoa repens*, tested at 10 and 100 µg/ml, inhibited the activity of 5α-reductase by 15 and 35%, respectively. At 1 µg/ml, it had no effect.

In conclusion, under the experimental conditions used, the extracts of soybean isoflavones and of *Serenoa repens* (reference) inhibited the activity of 5α-reductase. The extract of soybean isoflavones displays a 5α-reductase-inhibiting effect higher than that of the extract of *Serenoa repens*.

2.3. Extract of *Pygeum africanum*

In the control cultures, the rate of metabolization of testosterone was 9.71 +/- 0.77 pmoles of 5α-DHT formed in 22 hours per culture well (Table in paragraph 3.3.). This rate was in conformity with the results already obtained in the laboratory.

The effects of the test products were compared with those obtained in the presence of finasteride, used as a reference product in addition to the extract of Serenoa repens.

Finasteride, tested at 3 and 30 ng/ml, inhibited the activity of 5α-reductase by 36 and 65% (Table 1). This result was expected and validated the study.

The extract of *Serenoa repens*, tested at 10 and 100 µg/ml, inhibited the activity of 5α-reductase by 15 and 35%. At 1 µg/ml it had no effect (Table 2).

The extract of *Pygeum africanum*, tested at 1, 10 and 100 µg/ml, inhibited the activity of 5α-reductase by 33, 22 and 30%, respectively (Table 2).

In conclusion, the extracts of *Serenoa repens* and *Pygeum africanum* inhibited the activity of 5α-reductase almost identically.

3. Detailed tables of results

3.1. Effect of genistin and genistein on the activity of 5α-reductase in cultures of normal human dermal fibroblasts after 24 hours of incubation

Product	DMSO	Concentration (µg/ml)			
	1% (v/v)	0.1	1	10	100
Genistin	10.56	11.72	9.80	13.64	7.12
	12.00	11.16	9.88	14.12	8.84
	11.64	9.36	10.16	12.64	7.20
	11.40 +/- 0.75	**10.75 +/- 1.23**	**9.95 +/- 0.75**	**13.47*+/- 0.76**	**7.72*+/- 0.97**
	100	*94*	*87*	*118*	*68*
Genistein	10.56	8.04	7.52	7.96	4.24
	12.00	7.52	8.56	8.68	4.96
	11.64	7.72	7.006	6.886	4.166
	11.40 +/- 0.75	**7.76*+/- 0.26**	**7.69*+/- 0.79**	**7.84*+/- 0.91**	**4.45*+/- 0.44**
	100	*68*	*67*	*69*	*39*

The results are expressed in pmoles of 5α-DHT formed per culture well
Bold: average and standard deviation
Italics: percentage of "0.1% (v/v) DMSO" group
*: average significantly different from "0.1% (v/v) DMSO" group

3.2. Effect of extracts of *Serenoa repens* and soybean isoflavones (Genosten 4000) on the activity of 5α-reductase in cultures of normal human dermic fibroblasts after 22 hours of incubation

Product	DMSO 1% (v/v)	Concentration (µg/ml)		
		1	10	100
Serenoa repens	8.00 8.92 8.68 **8.56 +/- 0.48** *100*	7.72 9.20 8.08 **8.33 +/- 0.77** *98*	6.84 7.48 7.52 **7.28 *+/- 0.38** *85*	5.48 5.68 5.44 **5.53*+/- 0.13** *65*
Extract of soybean isoflavones (Genosten 4000)	8.00 8.92 8.68 **8.56+/-0.48** *100*		6.24 6.52 7.12 **6.62*+/- 0.45** *78*	7.04 7.12 7.12 **7.09*+/- 0.05** *83*

The results are expressed in pmoles of 5α-DHT formed per culture well
Bold: average and standard deviation
Italics: percentage of DMSO group
*: average significantly different from the DMSO group ($p<0.05$)

3.3. Effect of finasteride and extracts of *Serenoa repens* and *Pygeum africanum* on the activity of 5α-reductase in cultures of normal human dermal fibroblasts after 22 hours of incubation

3.3.1. Finasteride

Control	DMSO 1% (vv)	Finasteride (ng/ml)	
		3	30
9.24 9.28 10.60 9.71*+/- 0.77 *114*	8.00 8.92 8.68 8.53 +/- 0.48 *100*	5.52 5.84 5.00 5.45 *+/- 0.42 *64*	2.92 2.92 3.00 2.94*+/- 0.05 *35*

The results are expressed in pmoles of 5α-DHT formed per culture well
Bold: average and standard deviation
Italics: percentage of DMSO group
*: average significantly different from DMSO group ($p<0.05$)

3.3.2. Extracts of *Serenoa repens* and *Pygeum africanum*

Product	DMSO 1% (vv)	Concentration (µg/ml)		
		1	10	100
Serenoa repens	8.0 8.92 8.68 **8.53 +/- 0.48** *100*	7.72 9.20 8.08 **8.33 +/- 0.77** *98*	6.84 7.48 7.52 **7.28*+/- 0.38** *85*	5.48 5.68 5.44 **5.53*+/- 0.13** *65*
Pygeum africanum	8.0 8.92 8.68 **8.53 +/- 0.48** *100*	5.00 5.64 6.40 **5.68*+/- 0.70** *67*	6.36 7.28 6.20 **6.61*+/- 0.58** *78*	5.84 6.52 5.60 **5.99*+/- 0.48** *70*

The results are expressed in pmoles of 5α-DHT formed per culture well
Bold: average and standard deviation
Italics: percentage of DMSO group
*: average significantly different from DMSO group ($p<0.05$)

Example 3: Composition of a shampoo for oily hair

Water	q.s.p. 100,000
Sodium Lauroamphoacetate	14,000
Coco-Glucoside	10,000
Magnesium Laureth Sulfate	5,000
PEG-40 Glyceryl Cocoate	3,450
PEG-150 Distearate	1,850
Sodium Coceth Sulfate	1,050
Citric Acid	450
Disodium EDTA	300
Perfume	200
Methylparaben	160
Butylparaben	60
Soybean isoflavone	1,000
Pygeum Africanum	500

Example 4: Composition of an emulsion for oily skin

Water	q.s.p. 100
Di-C12-13 Alkyl Malate	10,000
Glycerol	5,000
PEG-5 Glyceryl Stearate	3,500
Glyceryl Stearate	1,500
Ceresin	1,500
PEG-40 Stearate	1,500

Sorbitan Stearate	1,000
Zinc PCA	1,000
Cetyl Alcohol	1,000
Polyacrylamide	1,000
C-13-14 Isoparaffin	500
Perfume	500
Piroctone Olamine	300
Laureth-7	125
Sodium Polyacrylate	65
Citrate of hydrogenated palm glycerides	40
Soybean isoflavone	2,000

Example 5: Composition of an emulsion for oily skin

Water	q.s.p. 100
Di-C12-13 Alkyl Malate	10,000
Glycerine	5,000
PEG-5 Glyceryl Stearate	3,500
Glyceryl Stearate	1,500
Ceresin	1,500
PEG-40 Stearate	1,500
Sorbitan Stearate	1,000
Zinc PCA	1,000
Cetyl Alcohol	1,000
Polyacrylamide	1,000
C-13-14 Isoparaffin	500
Perfume	500
Piroctone Olamine	300
Laureth-7	125
Sodium Polyacrylate	65
Citrate of hydrogenated palm glycerides	40
Pygeum Africanum	1,000
Salicylic acid	1,000

Claims

Use of at least one product chosen from the group constituted of the isoflavones, the extracts of the African plum tree, and their mixtures, for the preparation of a composition intended to inhibit the activity of 5α-reductase.

2. Use as in claim 1, characterized by the fact that the composition is intended to inhibit the type 1 isoenzyme and/or the type 2 isoenzyme of 5α-reductase.

3. Use as in claim 1 or 2, characterized by the fact that the product is chosen from among the synthetic or naturally-occurring isoflavones.

4. Use as in any one of the preceding claims, characterized by the fact that the product is chosen from among the synthetic or naturally-occurring isoflavones of the group constituted of genistin, daidzin, glycitin, acetyldaidzin, acetylgenistin, acetylglycitin, malonyldaidzin, malonylgenistin, malonylglycitin, 2,4,4'-trihydroxydeoxybenzoin (THB), daidzein, genistein, glycitein, formononectin, biochanin A, genistein-4'-O-glucoside, 2'-hydroxygenistein-7-O-glucoside, genistein-C-8-glucoside, and their mixtures.

Use as in any one of the preceding claims, characterized by the fact that the product is chosen from the group constituted of genistin, genistein, and their mixtures.

6. Use as in any one of the preceding claims, characterized by the fact that the product is chosen from among the extracts of soybean isoflavones.

7 Use as in any one of the claims to 5, characterized by the fact that the product is chosen from among the extracts of lupin isoflavones.

8. Use as in any one of the preceding claims, characterized by the fact that the product is used in a proportion of between about 0.001 and about 100% by weight, relative to the total weight of the composition.

9. Use as in any one of the preceding claims, characterized by the fact that the composition prepared contains a pharmaceutically, dermatologically or cosmetically acceptable excipient.

10. Use as in claim 9, characterized by the fact that the excipient is suitable for administration by an external topical route or by the rectal route.

Use as in any one of the preceding claims, characterized by the fact that the composition is intended for the treatment of cutaneous pathologies and/or disorders linked to a congenital or acquired hyperactivity of 5α-reductase.

12. Use as in any one of the claims 1 to 10, characterized by the fact that the composition is intended for the treatment of prostatic hypertrophy

13. Use as in any one of the claims to 10, characterized by the fact that the composition is intended for the treatment of prostatic adenoma.

14. Use as in any one of the claims to 10, characterized by the fact that the composition is intended for the treatment of acne.

15. Use as in any one of the claims to 10, characterized by the fact that the composition is intended for the treatment of hyperseborrhea.

16. Use as in any one of the claims to 10, characterized by the fact that the composition is intended for the treatment of alopecia.

17. Use as in any one of the claims to 10, characterized by the fact that the composition is intended for the treatment of hirsutism.

18. Method of cosmetic treatment of oily skin, characterized by the fact that to the skin is applied a cosmetic composition containing at least one product as defined in any one of the claims 1 and 3 to 7.

19. Method of cosmetic treatment of hair loss, characterized by the fact that to the scalp is applied a cosmetic composition containing at least one product as defined in any one of the claims 1 and 3 to 7.

20. Method of cosmetic treatment of excess hair, characterized by the fact that to the zones of the skin displaying excess hair is applied a cosmetic composition containing at least one product as defined in any one of the claims 1 and 3 to 7.

21 Method of cosmetic treatment as in any one of the claims 18 to 20, characterized by the fact that the said product is present in the composition in a proportion of between about 0.001 and about 100% by weight, relative to the total weight of the composition.

22. Method as in any one of the claims 18 to 21, characterized by the fact that the cosmetic composition contains in addition at least one cosmetically acceptable excipient.

23 Use of at least one product as defined in any one of the claims and 3 to 7, as an additive in a food for human beings or animals.

24. Use as in claim 23, characterized by the fact that the product is present in the food in a proportion of between about 0.001 and about 100% by weight, relative to the total weight of the food.

FRENCH REPUBLIC

NPI
NATIONAL PATENT INSTITUTE

PARTIAL PRELIMINARY SEARCH REPORT

established based on the last claims filed before the start of the search
See SUPPLEMENTARY SHEET (s)

National Register No.
FA 583255
FR 0000573

DOCUMENTS CONSIDERED TO BE PERTINENT		Relevant Claim(s)	CLASSIFICATION ATTRIBUTED TO INVENTION BY NPI
Category	Reference of document with indication, if necessary, of pertinent parts.		
X	WO 99 22728 A (ARCH DEV CORP; LIAO SHUTSUNG (US); HIIPAKKA RICHARD A (US)) 14 May 1999 (05/14/1999) * page 2, line 25 - page 3, line 30 * * claims 1-7 * Table 1, (compounds (10, 17) Table 7, (compounds 23, 31) * page 10, line 1 - page 15, line 26 * * page 21, line 31 - page 22, line 4 *	1-5, 8-24	A61K7/ 48 A61K7/ 06 A61K35/ 78 A61K31/ 353 A61P17/ 08 A61P17/ 14 A61p17/ 10
X	JP 10 059995 A (FUJIMOTO BROS : KK) 3 March 1998 (03/03/1998) * abridged *	1-3, 8-15, 18, 21-24	
X	DATABASE WPI Derwent Publications Ltd., London, GB; AN 1990-278125 XP002149039 "5-alfa reducatse inhibitors contg. Iso:flavone cpd." & JP 02 193920 A (KAO CORPORATION) * abridged *	1-3, 8, 9, 11, 14-18, 21-24	TECHNICAL AREAS SEARCHED (Int.CL7) A61K
X	US 5 543 146 A (PEREZ CARLOS) 6 August 1996 (08/06/1996) * column 2, line 11-16 * * column 3, line 22-28 * * claims; examples *	1-3, 8-10, 12 13, 22-24	
X	US 5 972 345 A (CHIZICK STEPHEN ET AL) 26 October 1999 (10/26/1999) * column 3, line 19-40; claims *	1-3, 8-11, 16, 19, 21-24	

Date of completion of International Search:	Examiner:
October 03, 2000	Veronese, A.

CATEGORY OF DOCUMENTS CITED:
- X: particularly pertinent on its own
- Y: particularly pertinent in combination with one or more other such documents in the same category.
- A: a technical background
- O: document referring to an oral disclosure.
- P: interposed document

- T: principle or theory underlying the invention
- E: patent document having a date earlier than the datre of filing and which was only published on this filing date or at a later date.
- D: document cited in the patent application
- L: document cited for other reasons

&: corresponding document, member of the same patent family

DOCUMENTS CONSIDERED TO BE PERTINENT		Relevant Claim(s)	CLASSIFICATION ATTRIBUTED TO INVENTION BY NPI
Category	Reference of document with indication, if necessary, of pertinent parts.		
X	DATABASE WPI Derwent Publications Ltd., London, GB; AN 1986-129442 XP002149040 "Hair tonic preparation showing hair-growing effects containing extract bark prunus africana." & JP 61 068408 A (POLA KASEY KOGYO KK), 1986 * abridged *	1-3, 8-11, 16, 19, 21-24	
P, X	SHIMIZU, KUNIYOSHI ET AI: "The 5-alpha.-reductase inhibitory components from heartwood of Artocarpus incisus. Structure-activity investigations" PLANTA MED. (2000)(, 66(1), pp. 16-19 , XP000951500 * Entire Document *	1-3, 8, 9, 12-16, 19-22	
			TECHNICAL AREAS SEARCHED (Int.CL7)

Date of completion of International Search:	Examiner:
October 03, 2000	Veronese, A.

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- L: document cited for other reasons

&: corresponding document, member of the same patent family

INCOMPLETE SEARCH
SUPPLEMENTARY SHEET 3

Appl. No.
FA 583255
FR 0000573

Certain claims have not been the subject of a search or have been the subject of an incomplete search, i.e.

Claims that were the subject of incomplete searches:
1-24

Reason:

The definitions "isoflavones" and "African plum tree" present in claim 1 relates to a very great variety of compounds/products. A foundation and/or a factual statement can however be found only for a very limited number of these compounds/products claimed. In the present case, the claims lack foundation to such an extent, and the factual statement on the invention in the description is so limited, that a significant search covering the whole spectrum claimed is impossible. Consequently, the search was limited to the parts of the claims that present a foundation and a factual statement, that is, the parts that have to do with the compounds mentioned in claim 4, in the description on page 5-6, and to the extracts of the African plum tree called "Pygeum" and Prunus africana."

The definition "composition intended to inhibit the activity of 5-alpha-reductase" present in claim 1 does not define the diseases for which the compositions are intended. Consequently, the search was limited to the diseases mentioned in claims 12-20.